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# NorM, a Putative Multidrug Efflux Protein, of *Vibrio parahaemolyticus* and Its Homolog in *Escherichia coli*

YUJI MORITA,[1] KAZUYO KODAMA,[1] SUMIKO SHIOTA,[1] TOMOYUKI MINE,[1] ATSUKO KATAOKA,[1] TOHRU MIZUSHIMA,[1] AND TOMOFUSA TSUCHIYA[1,2,*]

*Department of Microbiology, Faculty of Pharmaceutical Sciences,[1] and Gene Research Center,[2] Okayama University, Tsushima, Okayama 700-8530, Japan*

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**We found that cells of *Vibrio parahaemolyticus* possess an energy-dependent efflux system for norfloxacin. We cloned a gene for a putative norfloxacin efflux protein from the chromosomal DNA of *V. parahaemolyticus* by using an *Escherichia coli* mutant lacking the major multidrug efflux system AcrAB as the host and sequenced the gene (*norM*). Cells of *E. coli* transformed with a plasmid carrying the *norM* gene showed elevated energy-dependent efflux of norfloxacin. The transformants showed elevated resistance not only to norfloxacin and ciprofloxacin but also to the structurally unrelated compounds ethidium, kanamycin, and streptomycin. These results suggest that this is a multidrug efflux system. The hydropathy pattern of the deduced amino acid sequence of NorM suggested the presence of 12 transmembrane domains. The deduced primary structure of NorM showed 57% identity and 88% similarity with that of a hypothetical *E. coli* membrane protein, YdhE. No reported drug efflux protein in the sequence databases showed significant sequence similarity with NorM. Thus, NorM seems to be a novel type of multidrug efflux protein. We cloned the *ydhE* gene from *E. coli*. Cells of *E. coli* transformed with the cloned *ydhE* gene showed elevated resistance to norfloxacin, ciprofloxacin, acriflavine, and tetraphenylphosphonium ion, but not to ethidium, when MICs were measured. Thus, it seems that NorM and YdhE differ somehow in substrate specificity.**

Drug efflux from cells is one of the major mechanisms of drug resistance in bacteria. Many drug efflux systems are known in many bacteria (4, 22, 31). Four major groups of drug extrusion systems are known (4, 22, 31), i.e., the MF (major facilitator) family, the SMR (small multidrug resistance) family, the RND (resistance nodulation cell division) family, and the ABC (ATP binding cassette) family. Membrane transporters of the MF family possess 12 to 14 transmembrane domains. For example, Bcr (*Escherichia coli*) (1), EmrB (*E. coli*) (14), EmrD (*E. coli*) (20), NorA (*Staphylococcus aureus*) (34), QacA (*S. aureus*) (26), and Bmr (*Bacillus subtilis*) (21) are members of this family, and these systems mediate drug extrusion with different specificities. Transporters of the SMR family are rather small and usually possess four transmembrane domains. Smr (or QacC) (*S. aureus*) (8, 13), QacE (*Klebsiella aerogenes*) (23), and EmrE (*E. coli*) (33) belong to this family. An electrochemical potential of $H^+$ across cell membranes seems to be the driving force for drug efflux by the MF and SMR family transporters. Transporters of the RND family consist of several subunits (usually three), and an outer membrane protein(s) is involved in the drug transport. AcrAB (*E. coli*) (16) and MexAB (*Pseudomonas aeruginosa*) (24) are examples of members of this family. Energy coupling in this family is not very clear. However, it is clear that at least an electrochemical potential of $H^+$ across cell membranes is involved in driving the drug efflux (16). Transporters of the ABC family utilize ATP as the energy source. LmrA (*Lactococcus lactis*) (32) and MsrA (*S. aureus*) (25) are members of this family.

*Vibrio parahaemolyticus*, a slightly halophilic marine bacterium, is one of the major causes of food poisoning in Japan (18). Energy metabolism and energy coupling in membranes of this microorganism are unique (11). Cells of *V. parahaemolyticus* show some natural resistance to some antimicrobial agents. Thus, we were interested in drug efflux systems of *V. parahaemolyticus*. Cells of *V. parahaemolyticus* may possess unique drug efflux systems. During the course of our studies, we found that cells of *V. parahaemolyticus* possess an energy-dependent efflux system for norfloxacin, a widely used new quinolone antimicrobial agent. Here we report the cloning and sequencing of the gene for and characterization of a putative norfloxacin efflux protein of *V. parahaemolyticus* and the cloning and characterization of the homolog gene found in *E. coli*.

* Corresponding author. Mailing address: Department of Microbiology, Faculty of Pharmaceutical Sciences, Okayama University, Tsushima, Okayama 700-8530, Japan. Phone and Fax: 81-86-251-7957. E-mail: tsuchiya@pharm.okayama-u.ac.jp.

## MATERIALS AND METHODS

**Bacteria and growth.** *V. parahaemolyticus* AQ3334 (30) and *E. coli* TG1 [Δ(*lac-pro*) *supE thi hsd*Δ*5*/F′*traD36 proA*$^+$*B*$^+$ *lacI*$^q$ *lacZ*ΔM15], a derivative of K-12, and KAM2 and KAM3, derivatives of TG1, were used in this study. Cells of *V. parahaemolyticus* AQ3334 were grown in LB medium (17) under aerobic conditions at 37°C, and *E. coli* cells were grown in L medium (12) under aerobic conditions at 37°C. Where indicated, drugs were added to the medium. Cell growth was monitored turbidimetrically at 650 nm.

**Isolation of mutants.** KAM3, which has a deletion in the chromosomal *acrAB* genes, was obtained as follows. We tried to disrupt the *acrAB* genes, which code for the major multidrug efflux system of *E. coli*, by inserting the Mu phage. Cells of *E. coli* TG1 were infected with the Mud(Ap$^r$ *lac*)I phage (5, 29). Cells were diluted with L broth and spread onto an agar plate containing L broth and 40-μg/ml ampicillin. After incubation at 37°C for 12 h, colonies were picked up and replica plated by using (i) an agar plate containing L broth and 40-μg/ml ampicillin and (ii) an agar plate containing L broth, 40-μg/ml ampicillin, and 50-μg/ml methylene blue, an antimicrobial basic dye and substrate for the AcrAB system (16). We obtained two colonies which grew in the presence of methylene blue. One of them was designated KAM2. Thereafter, the Mu phage region was removed from the chromosome of KAM2 cells by heat induction at 42°C (5, 29), and colonies that were sensitive to both ampicillin and methylene blue were obtained. One of the colonies was designated KAM3. The KAM3 cells, as well as the KAM2 cells, were sensitive to many drugs that are known as substrates of the AcrAB system, although TG1 cells were resistant. The deletion in the *acrAB* region in KAM3 was confirmed by Southern blot analysis (data not shown).

**Assay of norfloxacin accumulation in cells.** *V. parahaemolyticus* cells were grown in the LB broth supplemented with 40 mM potassium lactate. The cells

were harvested at the exponential phase of growth, washed with 0.2 M MOPS-Tris buffer (pH 7.0) containing 10 mM $MgSO_4$, and suspended in the same buffer to 50 mg (wet weight)/ml. The assay mixture contained cells (10 mg [wet weight]/ml) in the same buffer and 10 mM potassium lactate. After incubation at 25°C for 5 min, norfloxacin (100 μM, final concentration) was added to initiate the assay. Samples (1 ml each) were taken at intervals, centrifuged at 7,000 × *g* for 30 s at 4°C, and washed once with the same buffer. Where indicated, carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) was added to the assay mixture at 100 μM. The pellet was suspended in 1 ml of 100 mM glycine-HCl (pH 3.0). The suspension was shaken vigorously for 1 h at room temperature and then centrifuged at 7,000 × *g* for 5 min at room temperature. The supernatant was diluted twofold with the same buffer, and the fluorescence was measured with excitation at 277 nm and emission at 448 nm (10, 19) with a Hitachi F2000 fluorescence spectrophotometer.

**Assay of ethidium accumulation in cells.** Cells were prepared and suspended as described above for the norfloxacin accumulation assay. Potassium lactate (final concentration 20 mM) was added to the cell suspension (0.5 mg of protein/ml), and the cell suspension was kept at 25°C for 5 min with gentle stirring. Ethidium bromide was added at 10 μM to the cell suspension to initiate the assay. CCCP was added at 100 μM where indicated. The fluorescence of the assay mixture was measured with excitation and emission wavelengths of 500 and 580 nm (3), respectively.

**Gene cloning and sequencing.** The gene responsible for norfloxacin efflux was cloned from *V. parahaemolyticus* cells as follows. Chromosomal DNA was prepared from cells of *V. parahaemolyticus* by the method of Berns and Thomas (2). The DNA was partially digested with *Sau*3AI, and fragments of 4 to 10 kbp were separated by sucrose density gradient centrifugation. The DNA fragments were ligated into pBR322 (which had been digested with *Bam*HI and dephosphorylated with bacterial alkaline phosphatase) by using T4 DNA ligase. Competent cells of *E. coli* KAM3 were transformed with the ligated hybrid plasmids and were spread on agar plates containing L broth, 0.05-μg/ml norfloxacin, 60-μg/ml ampicillin, and 1.5% agar. The plates were incubated at 37°C for 24 h, and the clones formed were picked up. Plasmids contained in the transformants were isolated, reintroduced into KAM3 cells, and spread on the plates again. The plates were incubated at 37°C for 24 h. Plasmids contained in the retransformants were prepared. One of the resulting hybrid plasmids that carried a gene for the norfloxacin efflux system was designated pMVP3.

The DNA insert in pMVP3 was digested with several restriction endonucleases and subcloned into pBR322. The resulting hybrid plasmids were introduced into KAM3 cells, and the transformants were tested for sensitivity or resistance to norfloxacin.

The nucleotide sequence was determined by the dideoxy chain termination method (27) with a DNA sequencer (ALF Express, Pharmacia Biotech).

The *ydhE* gene of *E. coli* was cloned as follows. Chromosomal DNA was prepared from cells of *E. coli* KAM3 by the method of Berns and Thomas (2). The DNA was digested with *Ssp*I and *Sph*I, which should cut out the whole *ydhE* gene (EMBL nucleotide sequence database accession no. AE000261), judging from the DNA sequence of the *E. coli* genome. The *Ssp*I-*Sph*I fragments were ligated into pBR322 (which had been digested with *Eco*RV and *Sph*I and dephosphorylated with bacterial alkaline phosphatase) by using T4 DNA ligase. Competent cells of *E. coli* KAM3, which are very sensitive to norfloxacin, were transformed with the ligated hybrid plasmids and spread on plates containing L broth, 0.05-μg/ml norfloxacin, 60-μg/ml ampicillin, and 1.5% agar. The plates were incubated at 37°C for 24 h, and the clones formed were picked up. Plasmids contained in the transformants were checked for whether they contained the expected *Ssp*I-*Sph*I fragment. One of the resulting hybrid plasmids that carried the *ydhE* gene was designated pMEC2.

Sequence data were analyzed with GENETYX sequence analysis software (Software Development Co.). The SwissProt and GenBank databases were screened for sequence similarities.

**Southern blot analysis.** Chromosomal DNAs were prepare from cells of *E. coli*, *V. parahaemolyticus*, *V. alginolyticus*, *P. aeruginosa*, and *S. aureus* as described above. The DNAs were digested with several restriction enzymes and subjected to agarose gel electrophoresis. DNA fragments were blotted onto a Hybond-N (Amersham Co.) nylon membrane by the capillary blotting method as suggested by the manufacturer. The probes used were three DNA fragments derived from the *acrRAB* genes of *E. coli*, which are shown below. Southern blot analysis was performed with the enhanced-chemiluminescence detection system (Amersham Co.) as suggested by the manufacturer.

**Drugs susceptibility test.** The MICs of drugs were determined in Mueller-Hinton broth (Difco) containing various drugs at various concentrations. Cells in the test medium were incubated at 37°C for 24 h, and growth was judged thereafter.

**Other.** Protein contents were determined by the method of Lowry et al. (15). The chemicals and enzymes used in this study were from commercial sources.

**Nucleotide sequence accession number.** The nucleotide sequence data reported in this paper have been deposited in the DDBJ, EMBL, and GenBank nucleotide sequence databases under accession no. AB010463.

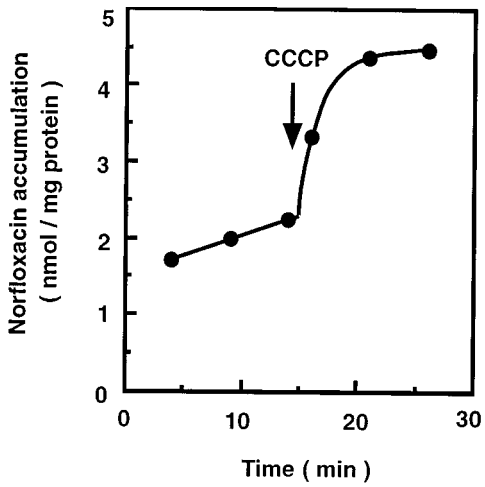


FIG. 1. Accumulation of norfloxacin in cells of *V. parahaemolyticus*. *V. parahaemolyticus* cells were grown in LB medium supplemented with 40 mM potassium lactate. Norfloxacin was added to the cell suspensions at a final concentration of 100 μM. After 15 min, CCCP was added to the suspensions at a final concentration of 100 μM. Portions were removed at the times shown, and the concentration of norfloxacin extracted from the cells was determined with a fluorescence spectrophotometer.

## RESULTS AND DISCUSSION

**Norfloxacin efflux in cells of *V. parahaemolyticus*.** We measured norfloxacin accumulation and tested the effect of an $H^+$ conductor, CCCP, on the accumulation in cells of *V. parahaemolyticus*. A certain level of norfloxacin accumulation in the cells was observed, and the accumulation level increased after addition of CCCP (Fig. 1), suggesting the existence of a norfloxacin efflux system in *V. parahaemolyticus* that is driven by an electrochemical potential of $H^+$. It is not clear whether $H^+$ is directly or indirectly coupled to the drug efflux.

**Cloning of the gene for a putative norfloxacin efflux protein.** We cloned a fragment of the chromosomal DNA of *V. parahaemolyticus*, which enabled norfloxacin-hypersensitive *E. coli* KAM3 cells to grow in the presence of norfloxacin. We obtained about 40 candidate hybrid plasmids. However, it seemed that the DNA inserts in all of the candidate plasmids contained the same DNA portion, judging from restriction maps of those plasmids. We tested whether the plasmids carry a gene(s) responsible for norfloxacin efflux or not by measuring norfloxacin accumulation and the effect of CCCP in *E. coli* cells transformed with the plasmids. A considerable level of norfloxacin accumulation was observed with the host cells (*E. coli* KAM3). Addition of CCCP increased the accumulation level to some extent, supporting the presence of some norfloxacin efflux system(s) in *E. coli* KAM3 (6). Cells harboring the plasmids showed very low levels of norfloxacin accumulation compared with those of the host cells. The norfloxacin accumulation in cells harboring one of the plasmids, pMVP3, is shown in Fig. 2. Addition of CCCP to the assay mixture greatly increased the accumulation level (Fig. 2). The norfloxacin accumulation level after the addition of CCCP was very similar in the host cells and the transformed cells. Thus, we conclude that the elevated norfloxacin efflux is due to the gene(s) carried on plasmid pMVP3.

Inhibition of the drug efflux in KAM3/pMVP3 cells by CCCP indicates that an electrochemical potential of $H^+$ is the driving force for the drug extrusion. Some drug/$H^+$ antiporters, such as the tetracycline/$H^+$ antiporter (9), are known in microbial cells. It is very likely that the norfloxacin extrusion

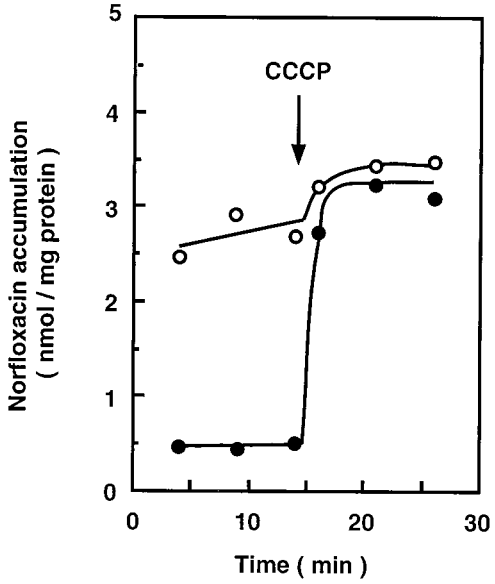


FIG. 2. Accumulation of norfloxacin in host and transformant cells. Cells of *E. coli* KAM3 and KAM3/pMVP3 were grown in L medium supplemented with 40 mM potassium lactate. Norfloxacin was added to the cell suspensions at a final concentration of 100 μM. After 15 min, CCCP was added to the suspensions at a final concentration of 100 μM. Portions were removed at the times shown, and the concentration of norfloxacin extracted from the cells was determined with a fluorescence spectrophotometer. Symbols: ○, KAM3; ●, KAM3/pMVP3.

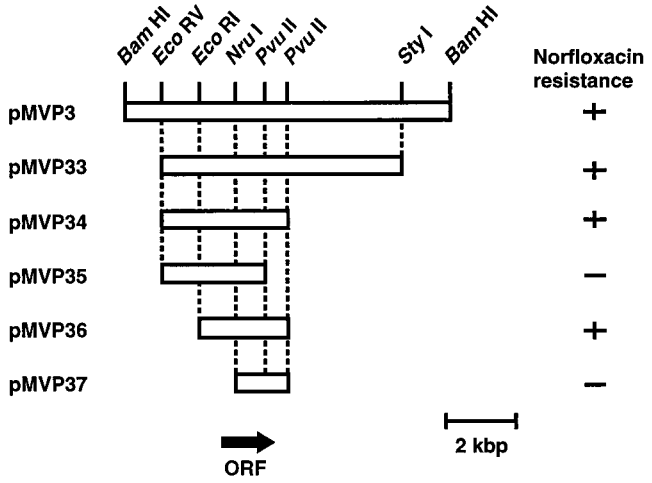


FIG. 3. Restriction maps of pMVP3 and its deletion derivatives. DNA regions derived from *V. parahaemolyticus* chromosomal DNA and carried by each plasmid are shown. Growth of *E. coli* KAM3 cells harboring each plasmid on an agar plate containing L broth, 0.05-μg/ml norfloxacin, and 60-μg/ml ampicillin is shown on the right. Plus signs indicate that cells grew, and minus signs indicate that cells did not grow. The arrow shows the position and direction of the *norM* gene. ORF, open reading frame.

system in KAM3/pMVP3 is a drug/ion antiporter. However, it is not clear whether this system utilizes $H^+$ as the counterion for the antiport. Many ion-coupled systems in membranes of vibrios utilize $Na^+$ instead of $H^+$ (28). Although cells of *V. parahaemolyticus* possess a primary respiratory $Na^+$ pump (30), $Na^+$-coupled membrane processes, such as $Na^+$/solute symport, are sensitive to an $H^+$ conductor (unpublished results). Therefore, we cannot distinguish between an $H^+$-coupled system and an $Na^+$-coupled system from the effects of an $H^+$ conductor. We tested the effect of $Na^+$ on the activity of norfloxacin extrusion in cells of both *V. parahaemolyticus* and *E. coli* transformed with pMVP3. However, no clear effect was observed (data not shown). We also tried to detect norfloxacin/$H^+$ antiport by the quinacrine fluorescence quenching method with everted membrane vesicles prepared from cells of KAM3/pMVP3. However, we were unable to detect $H^+$ efflux due to norfloxacin influx (data not shown).

Several plasmids carrying different portions of pMVP3 were constructed, and the ability to confer norfloxacin resistance on *E. coli* KAM3 was tested (Fig. 3). Among the plasmids that conferred norfloxacin resistance, plasmid pMVP36 possessed the shortest DNA insert derived from *V. parahaemolyticus* DNA (Fig. 3).

We tested the susceptibility of cells of KAM3/pMVP36 to many drugs (Table 1). pMVP36 made KAM3 cells resistant to rather hydrophilic new quinolones, such as norfloxacin and ciprofloxacin, but not to hydrophobic quinolones, such as spafloxacin and nalidixic acid. Cells harboring pMVP36 were also resistant to structurally different antimicrobial agents, such as ethidium and streptomycin (and kanamycin). Thus, it seems that pMVP36 carries a gene for multidrug resistance. Our results are consistent with the idea that the gene encodes a protein mediating the extrusion of norfloxacin and ethidium from cells. In fact, we observed elevated ethidium efflux in cells of KAM3/pMVP36 compared with cells of KAM3 (data not shown).

We checked whether the cloned DNA fragment is really from *V. parahaemolyticus*, and whether a similar gene is present in other bacteria, by Southern blot analysis. A DNA fragment derived from pMVP36 was used as a probe. We detected a dense hybridized band with chromosomal DNA of *V. parahaemolyticus* (data not shown). The position of the band was exactly the same as that of the control (pMVP36). Thus, we believe that the DNA insert in pMVP36 is from the chromosomal DNA of *V. parahaemolyticus*. We also detected a less dense band with chromosomal DNA of *V. alginolyticus*. Thus, it seems that cells of *V. alginolyticus* possess a similar gene. Chromosomal DNAs from other bacteria tested, *P. aeruginosa*

TABLE 1. Susceptibilities of study strains to different compounds

| Compound | MIC (μg/ml) | | |
|---|---|---|---|
| | KAM3 | KAM3/pMVP36 | KAM3/pMEC2 |
| Norfloxacin | 0.03 | 0.24 | 0.24 |
| Ciprofloxacin | 0.004 | 0.015 | 0.015 |
| Ofloxacin | 0.015 | 0.015 | 0.015 |
| Spafloxacin | 0.001 | 0.001 | 0.001 |
| Nalidixic acid | 0.5 | 0.5 | 0.5 |
| Ethidium | 2 | 16 | 2 |
| Acriflavine | 2 | 2 | 8 |
| Rhodamine 6G | 8 | 8 | 8 |
| Berberine | 64 | 128 | ≥168 |
| Quinacrine | 128 | 128 | 128 |
| Tetraphenylphosphonium ion | 8 | 8 | 64 |
| Tetrachlorosalicylanilide | 8 | 8 | 8 |
| CCCP | 8 | 8 | 8 |
| Kanamycin | 1 | 4 | 2 |
| Streptomycin | 1 | 4 | 2 |
| Erythromycin | 1 | 1 | 1 |
| Tetracycline | 0.25 | 0.25 | 0.25 |
| Chloramphenicol | 1 | 1 | 1 |
| Sodium dodecylsulfate | 64 | 64 | 64 |

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NorM VPARA   1:M-----HRYKEEASSLIKLATPVLIASVAQTGMGFVDTVMAGGVTQTDMAAVSVASSIWL 55
               *     ..* .**. *. **.**..* .***.****.****** ..******....****
YdhE ECOLI   1:M-----QKYISEARLLLALAIPVILAQIAQTAMGFVSTVMAGGYSATDMAAVAIGTSIWL 55
YdhE HAEIN   1:MNFRLLSQYHADIKKLIKISLPILLAQIAQNSMGLADTIMAGRVSSTDMAAISIGASIWM 60

NorM VPARA  56:PSILFGIGLLMALVPVVAQLNGSARREKIPFEIQQGVVLALLISIPIIGVLLQTQFILQL 115
               *.**** ***.**.**.******.***.*. ...**  ** ..*. *. ** .. .*..
YdhE ECOLI  56:PAILFGHGLLLALTPVIAQLNGSGRRERIAHQVRQGFWLAGFVSVLIMLVLWNAGYIIRS 115
YdhE HAEIN  61:PLMFFGQGLLLALPPTISYLNGSGQHHRIAHQVRQGIWLVLGVSIPL-GLLIYFCEIPLQ 119

NorM VPARA 116:MD-VEAVMADKTVGYIHAVIFAVPAFLLFQTLRSFTDGMSLTKPAMVIGFIGLLLNIPLN 174
               *. .....***.***..*.....*..*.**. *.  .*.. ***.**.******.***.*
YdhE ECOLI 116:MENIDPALADKAVGYLRALLWGAPGYLFFQVARNQCEGLAKTKPGMVMGFIGLLVNIPVN 175
YdhE HAEIN 120:YMQMESKMSDLARDYLHAMLWGLPAYLMLINFRCLNDGIEKTKPAMVITFLGLLLINIPLN 179

NorM VPARA 175:WIFVYGKFGAPELGGVGCGVATTIVYWVMFALLLAYVMTSSRLKSI-NVFGEYHKPQWKA 233
               .**.**.** ************. ******  ...*.. ......* .   .  **.  .
YdhE ECOLI 176:YIFIYGHFGMPELGGVGCGVATAAVYWVMFLAMVSYIKRARSMRDI-RNEKGTAKPDPAV 234
YdhE HAEIN 180:YIFIYGKFGMPAFGAVGCGIATAIVNWAMCLMMIFYSYTNTQERSLKVFSQLIEMPNPKT 239

NorM VPARA 234:QVRLFKLGFPVAAALFFEVTLFAVVALLVSPLGPIIVAAHQVAINFSSLVFMLPMSVGAA 293
                 **..**.*.* ********************* . **.**.*.*****.*.****..**
YdhE ECOLI 235:MKRLIQLGLPIALALFFEVTLFAVVALLVSPLGIVDVAGHQIALNFSSLMFVLPMSLAAA 294
YdhE HAEIN 240:LKKLLRLGLPIAIAICCEVALYALTSLMLSPLGATIVASHQITLNTSSFIFMFPMSIGMA 299

NorM VPARA 294:VSIRVGHRLGEENVDGARVASRVGIMVGLALATITAIITVLSRELIAELYTNNPEVITLA 353
               *.****.***.... .*..*.*.*.***. .**.***.**  ** ** **..****.***
YdhE ECOLI 295:VTIRVGYRLGQGSTLDAQTAARTGLMVGVCMATLTAIFTVSLREQIALLYNDNPEVVTLA 359
YdhE HAEIN 300:TTILVGQALGAGSPQNAKKIGYAALLLGLTVTIVTALITIFFRYEIASIFVTDEIVIAMA 359

NorM VPARA 354:MQLLLFAAVYQCTDAVQVIAAGALRGYKDMRAIFNRTFIAYWILGLPTGYILGRTDWIVE 413
               .*.*.***** .*..***..* ****** *.**  **.***.****.****. ** .**
YdhE ECOLI 355:AHLMLLAAVYQISDSIQVIGSGILRGYKDTRSIFYITFTAYWVLGLPSGYILALTDLVVE 414
YdhE HAEIN 360:ANLLLFAALYQFSDTIQMVVGGILRGYKDTKVILYITLFSYWVIGVPLGYTLGRTDWLVP 419

NorM VPARA 414:PMGAQGFWLGFIIGLTAAALMLGVRLRWMHRQEPDVQLNFSLQ--                456
               ***..***.*******.**.*. .*.*...*  ... *.
YdhE ECOLI 415:PMGPAGFWIGFIIGLTSAAIMMMLRMRFLQRLPSAIILQRASR--                457
YdhE HAEIN 420:HIDAKGFWIAFVVSLTFAAFLLSLRMKKMQAMNDNAILQRLEKLK                464
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FIG. 4. Amino acid sequence alignment. The deduced amino acid sequence of *V. parahaemolyticus* NorM (NorM VPARA) and the amino acid sequences of *E. coli* YdhE (YdhE ECOLI) and *H. influenzae* YdhE (YdhE HAEIN) are aligned. The numbers on both sides refer to the position of the nearest residue on each line. Asterisks and dots indicate residues in YdhE of *E. coli* that are identical and similar to those in NorM, respectively.

and *S. aureus*, showed no hybridized band under our experimental conditions.

**Gene and protein sequences.** We determined the sequence of 2,243 nucleotides of the DNA insert in pMVP36. We found only one open reading frame, which was preceded by a Shine-Dalgarno sequence in this region, which is likely to be the gene (which we designated *norM*) encoding the norfloxacin efflux protein (NorM). Several promoterlike sequences were present upstream of *norM*. The amino acid sequence deduced from the *norM* gene revealed that NorM consists of 456 amino acid residues (Fig. 4) with a molecular mass of 49,422 Da and is very rich in hydrophobic residues, indicating that the protein is a membrane protein. A hydropathy analysis done by the method of Eisenberg et al. (7) revealed that NorM possesses 12 hydrophobic regions which may be transmembrane domains (data not shown). Therefore, it is likely that NorM is a member of the MF family.

**Homolog of NorM.** We searched for amino acid sequence homology between NorM and the reported sequences in a protein sequence database (SwissProt). No sequence homology

or similarity was observed between NorM and the reported members of the MF family. The first extrusion system for norfloxacin to be reported in microbial cells is the NorA system of *S. aureus* (34). We found no sequence similarity between NorM and NorA. However, two hypothetical proteins, YdhE of *E. coli* and that of *Haemophilus influenzae*, showed high sequence similarity with NorM. The sequence identity and similarity between NorM and YdhE of *E. coli* were 57 and 88%, respectively, and those between NorM and YdhE of *H. influenzae* were 43 and 85%, respectively. Figure 4 shows an alignment of the deduced amino acid sequences of NorM and these YdhE proteins. Judging from the high sequence identity and similarity, it seems that NorM and these two YdhE proteins are homologs.

We cloned the *ydhE* gene from the chromosomal DNA of *E. coli* KAM3. Cells of *E. coli* KAM3 harboring a hybrid plasmid carrying the *ydhE* gene, pMEC2, showed elevated resistance to several drugs, such as norfloxacin, ciprofloxacin, and acriflavine, and the tetraphenylphosphonium ion, judging from the MICs (Table 1). Some increase in the MICs of kanamycin and streptomycin was observed. Surprisingly, no MIC increase was observed with ethidium, which is a good substrate for the NorM system.

Another hypothetical protein (YojI) of *B. subtilis* (EMBL database accession no. Z99114) had an amino acid sequence that was similar to that of NorM (35% identity and 77% similarity).

## ACKNOWLEDGMENTS

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## REFERENCES

1. **Bentley, J., L. S. Hyatt, K. Ainley, J. H. Parish, R. B. Herbert, and G. R. White.** 1993. Cloning and sequence analysis of an *Escherichia coli* gene conferring bicyclomycin resistance. Gene **127:**117–120.
2. **Berns, K. I., and C. A. J. Thomas.** 1965. Isolation of high molecular weight DNA from *Haemophilus influenzae*. J. Mol. Biol. **11:**476–490.
3. **Bolhuis, H., D. Molenaar, G. Poelarends, H. W. van Veen, B. Poolman, A. J. M. Driessen, and W. N. Konings.** 1994. Proton motive force-driven and ATP-dependent drug extrusion systems in multidrug-resistant *Lactococcus lactis*. J. Bacteriol. **176:**6957–6964.
4. **Bolhuis, H., H. W. van Veen, B. Poolman, A. J. Driessen, and W. N. Konings.** 1997. Mechanisms of multidrug transporters. FEMS Microbiol. Rev. **21:**55–84.
5. **Casadaban, M. J., and S. N. Cohen.** 1979. Lactose genes fused to exogenous promoters in one step using a Mu-*lac* bacteriophage: in vivo probe for transcriptional control sequences. Proc. Natl. Acad. Sci. USA **76:**4530–4533.
6. **Cohen, S. P., D. C. Hooper, J. S. Wolfson, K. S. Souza, L. M. McMurry, and S. B. Levy.** 1988. Endogenous active efflux of norfloxacin in susceptible *Escherichia coli*. Antimicrob. Agents Chemother. **32:**1187–1191.
7. **Eisenberg, D., E. Schwarz, M. Komaromy, and R. Wall.** 1984. Analysis of membrane and surface protein sequences with the hydrophobic moment plot. J. Mol. Biol. **179:**125–142.
8. **Grinius, L., G. Dreguniene, E. B. Goldberg, C. H. Liao, and S. J. Projan.** 1992. A staphylococcal multidrug resistance gene product is a member of a new protein family. Plasmid **27:**119–129.
9. **Kaneko, M., A. Yamaguchi, and T. Sawai.** 1985. Energetics of tetracycline efflux system encoded by Tn10 in *Escherichia coli*. FEBS Lett. **193:**194–198.
10. **Kotera, Y., M. Watanabe, S. Yoshida, M. Inoue, and S. Mitsuhashi.** 1991. Factors influencing the uptake of norfloxacin by *Escherichia coli*. J. Antimicrob. Chemother. **27:**733–739.
11. **Kuroda, T., T. Shimamoto, K. Inaba, T. Kayahara, M. Tsuda, and T. Tsuchiya.** 1994. Properties of the $Na^+/H^+$ antiporter in *Vibrio parahaemolyticus*. J. Biochem. **115:**1162–1165.
12. **Lennox, E. S.** 1955. Transduction of linked genetic characters of host by bacteriophage P1. Virology **1:**190–206.
13. **Littlejohn, T. G., D. DiBerardino, L. J. Messerotti, S. J. Spiers, and R. A. Skurray.** 1991. Structure and evolution of a family of genes encoding antiseptic and disinfectant resistance in *Staphylococcus aureus*. Gene **101:**59–66.
14. **Lomovskaya, O., and K. Lewis.** 1992. Emr, an *Escherichia coli* locus for multidrug resistance. Proc. Natl. Acad. Sci. USA **89:**8938–8942.
15. **Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall.** 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. **193:**265–275.
16. **Ma, D., D. N. Cook, M. Alberti, N. G. Pon, H. Nikaido, and J. E. Hearst.** 1993. Molecular cloning and characterization of *acrA* and *acrE* genes of *Escherichia coli*. J. Bacteriol. **175:**6299–6313.
17. **Maniatis, T., E. F. Fritsch, and J. Sambrook.** 1982. Molecular cloning: a laboratory manual, p. 68. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
18. **Miwatani, T., and Y. Takeda.** 1976. Food poisoning due to *Vibrio parahaemolyticus* in Japan, p. 22–25. *In* T. Miwatani and Y. Takeda (ed.), *Vibrio parahaemolyticus*, a causative bacterium of food poisoning. Saikon Publishing Co., Tokyo, Japan.
19. **Mortimer, P. G., and L. J. Piddock.** 1991. A comparison of methods used for measuring the accumulation of quinolones by *Enterobacteriaceae*, *Pseudomonas aeruginosa* and *Staphylococcus aureus*. J. Antimicrob. Chemother. **28:**639–653.
20. **Naroditskaya, V., M. J. Schlosser, N. Y. Fang, and K. Lewis.** 1993. An *E. coli* gene *emrD* is involved in adaptation to low energy shock. Biochem. Biophys. Res. Commun. **196:**803–809.
21. **Neyfakh, A. A., V. E. Bidnenko, and L. B. Chen.** 1991. Efflux-mediated multidrug resistance in *Bacillus subtilis*: similarities and dissimilarities with the mammalian system. Proc. Natl. Acad. Sci. USA **88:**4781–4785.
22. **Nikaido, H.** 1996. Multidrug efflux pumps of gram-negative bacteria. J. Bacteriol. **178:**5853–5859.
23. **Paulsen, I. T., T. G. Littlejohn, P. Rådström, L. Sundström, O. Sköld, G. Swedberg, and R. A. Skurray.** 1993. The 3′ conserved segment of integrons contains a gene associated with multidrug resistance to antiseptics and disinfectants. Antimicrob. Agents Chemother. **37:**761–768.
24. **Poole, K., D. E. Heinrichs, and S. Neshat.** 1993. Cloning and sequence analysis of an EnvCD homologue in *Pseudomonas aeruginosa*: regulation by iron and possible involvement in the secretion of the siderophore pyoverdine. Mol. Microbiol. **10:**529–544.
25. **Ross, J. I., E. A. Eady, J. H. Cove, W. J. Cunliffe, S. Baumberg, and J. C. Wootton.** 1990. Inducible erythromycin resistance in staphylococci is encoded by a member of the ATP-binding transport super-gene family. Mol. Microbiol. **4:**1207–1214.
26. **Rouch, D. A., D. S. Cram, D. DiBerardino, T. G. Littlejohn, and R. A. Skurray.** 1990. Efflux-mediated antiseptic resistance gene *qacA* from *Staphylococcus aureus*: common ancestry with tetracycline- and sugar-transport proteins. Mol. Microbiol. **4:**2051–2062.
27. **Sanger, F., S. Nicklen, and A. R. Coulson.** 1977. DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. USA **74:**5463–5467.
28. **Skulachev, V. P.** 1989. The sodium cycle: a novel type of bacterial energetics. J. Bioenerg. Biomembr. **21:**635–647.
29. **Tate, C. G., J. A. Muiry, and P. J. Henderson.** 1992. Mapping, cloning, expression, and sequencing of the *rhaT* gene, which encodes a novel L-rhamnose-$H^+$ transport protein in *Salmonella typhimurium* and *Escherichia coli*. J. Biol. Chem. **267:**6923–6932.
30. **Tsuchiya, T., and S. Shinoda.** 1985. Respiration-driven $Na^+$ pump and $Na^+$ circulation in *Vibrio parahaemolyticus*. J. Bacteriol. **162:**794–798.
31. **van Veen, H. W., and W. N. Konings.** 1997. Drug efflux proteins in multidrug resistant bacteria. Biol. Chem. **378:**769–777.
32. **van Veen, H. W., K. Venema, H. Bolhuis, I. Oussenko, J. Kok, B. Poolman, A. J. Driessen, and W. N. Konings.** 1996. Multidrug resistance mediated by a bacterial homolog of the human multidrug transporter MDR1. Proc. Natl. Acad. Sci. USA **93:**10668–10672.
33. **Yerushalmi, H., M. Lebendiker, and S. Schuldiner.** 1995. EmrE, an *Escherichia coli* 12-kDa multidrug transporter, exchanges toxic cations and $H^+$ and is soluble in organic solvents. J. Biol. Chem. **270:**6856–6863.
34. **Yoshida, H., M. Bogaki, S. Nakamura, K. Ubukata, and M. Konno.** 1990. Nucleotide sequence and characterization of the *Staphylococcus aureus norA* gene, which confers resistance to quinolones. J. Bacteriol. **172:**6942–6949.